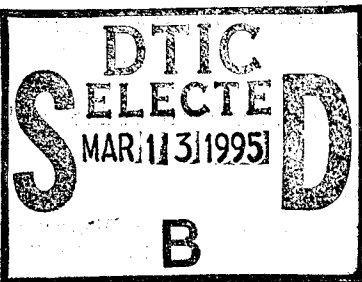


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**LIMITING FACTORS, ENHANCEMENT AND  
KINETICS OF BIODEGRADATION**

**FINAL REPORT**

Martin Alexander

January 1995

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The general objectives of the research were to (a) establish some of the limiting factors that prevent introduced microorganisms from biodegrading organic pollutants, (b) devise means to overcome these constraints and thus favor destruction of organic pollutants, (c) develop and evaluate new kinetic models for the biodegradation of organic pollutants and (d) determine the mechanisms by which microorganisms degrade organic pollutants not present in the aqueous phase.

Fresh and marine waters, sewage, and soils possess highly diverse microbial communities that exhibit many degradative capacities, and species within these communities destroy many organic chemicals. Nevertheless, many synthetic compounds persist in these environments even though the molecules are biodegradable, and hence, inoculation with species possessing the appropriate catabolic properties has been proposed as a means of enhancing the decomposition of these chemicals.

One possible reason for the failure of inoculation of contaminated sites with species able to degrade the chemical in culture is the inoculum size. A bacterium in axenic culture in rich medium is able to proliferate extensively even from a small inoculum. However, the low cell density that realistically can be introduced into large volumes of chemically polluted waters or soils may not replicate enough to bring about extensive biodegradation because other species inhabiting those environments may compete for the limited supply of inorganic nutrients or may prey on or parasitize the introduced population of bacteria before it becomes sufficiently large to bring about appreciable destruction of the polluting chemical.

Hence, a study was conducted to determine the importance of inoculum size in limiting the success of inocula added to lake water to bring about biodegradation. The test species was a strain of *Pseudomonas cepacia* able to grow on and mineralize 10 ng to 30  $\mu\text{g}$  of *p*-nitrophenol (PNP) per ml in salts solution. When introduced into water from Beebe Lake at densities of 330 cells per ml, *P. cepacia* did not mineralize 1.0  $\mu\text{g}$  of PNP per ml. However, PNP was mineralized in lake water inoculated with  $3.3 \times 10^4$  to  $3.6 \times 10^5$  *P. cepacia* cells per ml. In lake water containing 1.0  $\mu\text{g}$  of PNP per ml, a *P. cepacia* population of 230 or 120 cells per ml declined until no cells were detectable at 13 h, but when the initial density was  $4.3 \times 10^4$  cells per ml, sufficient survivors remained after the initial decline to multiply at the expense of PNP. The decline in bacterial abundance coincided with multiplication of protozoa. Cycloheximide and nystatin killed the protozoa and allowed the bacterium to multiply and mineralize 1.0  $\mu\text{g}$  of PNP, even when the initial *P. cepacia* density was 230 or 360 cells per ml. The lake water contained few lytic bacteria. The addition of  $\text{KH}_2\text{PO}_4$  or  $\text{NH}_4\text{NO}_3$  permitted biodegradation of PNP at low cell densities of *P. cepacia*. Thus, species able to degrade a synthetic chemical in culture may fail to bring about the same transformation in natural waters, because small populations added as inocula may be eliminated by protozoan grazing or may fail to survive because of nutrient deficiencies.

Another reason for the failure of inoculation is that the concentration of the compound is too low to support the growth of populations responsible for degrading the chemical. Below the threshold concentration for growth, i.e., the concentration of a substrate below which an organism cannot multiply, a substrate may not be metabolized and the bacterial cells may die.

The existence of a threshold concentration of a substrate for bacterial multiplication has been attributed to the fact that the energy source of the organism enters the cell at low substrate concentrations at a rate too slow to satisfy the need of the cells for energy to maintain viability. Because the energy requirements for maintenance vary among bacteria, threshold values are expected to differ among species. Indeed, marked differences in the threshold concentration among bacterial species have been reported. These differences in threshold concentrations for growth suggest that it may be possible to select inocula for the biodegradation of low levels of organic compounds, the organism with a lower minimum concentration for growth presumably being better able to metabolize low concentrations of a compound than an organism with a high threshold.

A study was thus conducted to determine whether bacteria with different thresholds for growth have dissimilar thresholds for biodegradation in culture and whether the organisms with the lower thresholds in culture would be able to degrade low concentrations of a test compound in samples of a natural environment. The test compound was PNP. In the investigation, we determined the effect of low concentrations of PNP on growth of four PNP-degrading bacteria and their abilities to metabolize low concentrations of the compound in culture and samples from a oligotrophic lake. PNP did not increase the growth rates of *Flavobacterium* sp. M4, *Pseudomonas* sp. K, *Flavobacterium* sp. M1, and *Pseudomonas* sp. SP3 at concentrations of less than 2, 4, 10, and 100 ng/ml, respectively, when it was the sole added carbon source in culture, but it stimulated multiplication at higher concentrations. In liquid culture with the nitro compound as sole added carbon source, the four bacteria extensively mineralized PNP at 50 and 100 ng/ml, and three of the four degraded much of the substrate at 25 ng/ml. *Pseudomonas* sp. SP3 mineralized more than 20% but the two *Flavobacterium* strains converted less than 10% of the substrate to CO<sub>2</sub> at 10 ng/ml, and none of the three mineralized more than 5% at 1 and 5 ng PNP/ml. Under conditions where more than 99% of the radioactivity from <sup>14</sup>C-PNP added at 1 ng/ml remained in solution, two of the isolates formed organic products. *Pseudomonas* sp. K had no activity at 1, 5, and 10 ng/ml. In contrast, when each of the bacteria was separately inoculated into samples of water from an oligotrophic lake and from a well in which PNP was not biodegraded, the bacteria were able to mineralize as little as 1 ng PNP/ml. The addition to a salts solution of 10 ng of glucose per ml resulted in mineralization of PNP at concentrations too low to be mineralized when the nitro compound was the sole source of added carbon. Bacteria may thus be able to mineralize substrates in natural waters at concentrations below those

suggested by tests conducted in culture media, possibly because of the availability of other carbon sources for the bacteria.

In considering inoculation of soils or subsoils, a major issue of concern is the distance between the pollutants and the site of adding the microorganisms. Should the contaminant be distant from the inoculum, the microorganisms must be capable of moving or being transported to it. Although much is known about factors affecting bacterial transport in soil, little information exists on degradation effected by bacteria inoculated at a distance from the substrate.

Because of the possibility that an organism added to the surface of soil may be retained by physical filtration or sorption and not reach an organic compound it can potentially metabolize, a study was conducted to determine the role of bacterial transport in the biodegradation of phenanthrene in columns of soil and aquifer sand. A phenanthrene-utilizing *Pseudomonas* sp. was introduced into the columns, either at a distance from or at the same location as the chemical, and three regimes of water flow were used. Addition of the bacterium to the surface of 1.5-cm columns of sterile or nonsterile soil or aquifer sand resulted in the rapid mineralization of phenanthrene present in the top 0.4 cm of sand or soil columns receiving intermittent or no water flow. However, the surface-inoculated pseudomonad mineralized little of the substrate present in the bottom 0.4-cm portion of nonsterile soil or aquifer sand that had been sterilized prior to inoculation, although mineralization occurred at the bottom of nonsterile aquifer sand. Little biodegradation was evident if the bacterium and the substrate were both at the bottom part of columns of soil and aquifer sand receiving intermittent flows of water, although rapid biodegradation occurred at this site in soil if the water flow was constant. Only approximately 1% of the added cells passed through the soil after passage of ca 1.5 pore volumes of water, although approximately 4% passed through the aquifer sand. It thus appears that bacteria added to the soil surface or to aquifer solids for bioremediation may not be transported sufficiently to reach organic pollutants at sites distant from channels or macropores.

The biodegradation of organic compounds is often slow because one or more organic nutrients (particularly P and N) that are needed for microbial growth are in low concentrations in natural environments. Because the addition of P sometimes increases the rate of biodegradation, it is important to be able to predict the concentration required to bring about the stimulation in natural environments. Such predictions are difficult because P precipitates with di- and trivalent cations, its solution chemistry is affected by the cations present, and the pH affects both the ionic species of P in solution and the identity of the soluble products of reactions with cations.

Information on reactions that affect phosphate availability to microorganisms is scant. Natural waters may be rich in Ca and Mg, and soils and sediments often

contain reactive Ca, Fe, and Mg. Such cations may alter the availability of P to microorganisms in ways analogous to how they affect P availability to rooted plants. Furthermore, the relative abundance of mono- and dibasic P as well as the identities of the Ca, Fe, and other salts varies with the pH values of natural environments. Hence, a study was conducted to determine some of the factors affecting the P requirement for the biodegradation of PNP, phenol, and glucose by *Pseudomonas* and *Corynebacterium* strains. Mineralization of glucose was rapid and the *Pseudomonas* sp. grew extensively in solutions with 5 and 10 mM phosphate, but the rate and extent of degradation were low and the bacterial population never became abundant in media with 0.2 mM phosphate. Similar results were obtained with the *Corynebacterium* sp. growing in media containing PNP or phenol and in solutions with a purified phosphate salt. The extent of growth of the *Corynebacterium* sp. was reduced with 2 or 10 mM phosphate in media containing high Fe concentrations. Ca at 5 mM but not 0.5 mM inhibited mineralization by the *Corynebacterium* sp. with phosphate concentrations from 0.2 to 5.0 mM. Phenol mineralization by the *Pseudomonas* sp. in medium with 0.2 mM phosphate was rapid at pH 5.2, but the bacteria had little or no activity at pH 8.0. In contrast, the activity was greater at pH 8.0 than at pH 5.2 when the culture contained 10 mM phosphate. These effects of pH were similar in media with 5 mM Ca or no added Ca. Thus the effect of P on bacterial degradation can be influenced by the pH and the concentrations of Fe and Ca.

The addition of inorganic nutrients does not always stimulate degradation because other factors may themselves suppress microbial activity or interact with nutrient limitation to slow degradation. Nutrient limitation may be exacerbated by other species using the same elements as those organisms metabolize other organic compounds. The presence of other bacteria or other substrates may reduce the degradation of test compounds. The mechanisms by which other microorganisms and other substrates affect biodegradation are presently unknown.

Inasmuch as biodegradation commonly occurs in environments containing several metabolizable organic substrates and frequently in circumstances where the supply of one or more inorganic nutrients is less than the demand, a study was undertaken to determine the effects of one species acting on a substrate on the biodegradation of a second substrate by a second species at low concentrations of P. In addition, experiments were conducted using lake water to assess the effects of limiting inorganic nutrients and second carbon sources.

Little benzylamine was found to be mineralized by *Pseudomonas putida* in solutions with no added P, but the substrate was degraded if the medium contained 0.1  $\mu$ M P. The enhancement by P addition did not occur if the medium also contained caprolactam and a caprolactam-utilizing strain of *Pseudomonas aeruginosa*. The suppression by the second bacterium was overcome by a higher P concentration. The rate of caprolactam utilization by *P. aeruginosa* was reduced if



benzylamine and *P. putida* were also present in media with 0.1  $\mu$ M P, but the suppression was absent if the solution contained a higher P concentration. Glutamate increased and inorganic N plus P decreased the length of the acclimation phase prior to benzylamine mineralization in lake water. The data suggest that the effect of one biodegradable substrate on the metabolism of a second often results from a competition for inorganic nutrients.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous, and some are environmental pollutants. Although many microorganisms can degrade PAHs in pure culture, the rate of their biodegradation in soils and sediments is slow. The slower rate of biodegradation in such environments than in culture suggests that microbial activity is being constrained in these natural environments. In some environments, the concentration of inorganic nutrients may be less than optimal for microbial proliferation or metabolism, and the addition of P and N sometimes results in enhancement of microbial growth and biodegradation of certain organic compounds. On the other hand, PAHs are sorbed by organic constituents of soil and sediments, and it is known that at least some sorbed substrates are more resistant to degradation than the nonsorbed chemicals.

An investigation was thus designed to study factors limiting the biodegradation of PAHs in soil. Phenanthrene was chosen as a model compound. Phenanthrene mineralization was enhanced by additions of phosphate but not potassium, and it was reduced by additions of nitrate. Aeration or amending the soil with glucose affected the rate of mineralization, although not markedly. Phenanthrene was sorbed to soil constituents, the extent of sorption being directly related to the percentage of organic matter in the soil. Soluble phenanthrene was not detected after addition of the compound to a muck soil. The rate of mineralization was slow in the organic soil and higher in mineral soils with lower percentages of organic matter. The data show that sorption by soil organic matter slows the biodegradation of polycyclic aromatic hydrocarbons that are otherwise readily metabolized.

A study also was conducted to determine the effect of sorption on the rate of biodegradation in lake sediments. In addition, polyacrylamide and polyvinylstyrene beads were used as model sorbents. Radiolabeled biphenyl, a compound which sorbs strongly to beads and sediments, and PNP, which sorbs moderately to beads and poorly to sediments, were used to investigate the effects of sorption and to distinguish between the effects of sorption and other influences of sediment on rates of degradation.

The removal of organic matter from the sediment decreased the rate of degradation of biphenyl by *Pseudomonas* sp. inoculated into sterile sediments. The rate of degradation of biphenyl by *Pseudomonas* sp. was higher when the bacteria were added to nonsterile than to sterile sediments. Organic carbon derived from

sediments increased the size of the population of *Pseudomonas* sp. growing on biphenyl but failed to increase the degradation rate of biphenyl sorbed to beads. Addition of biphenyl to sediments enhanced subsequent degradation of biphenyl sorbed to beads by the sediment bacteria.

The degradation of 100 ng of PNP sorbed to beads by a large population of *Corynebacterium* sp. was shown to be best described by a two-compartment model. A simulation model which predicts mineralization rates as a function of microbial activity in solution and desorption rates was developed. When compared with data from experiments using beads as sorbents, the model accurately predicted the mineralization curve for bacteria growing in the presence of sorbed phenol or PNP, but underrepresented the rate of mineralization when degradation was first-order.

*Pseudomonas* sp. K mineralized PNP faster when the bacteria were able to contact the sorbing surface than when the sorbed compound was within a dialysis bag with molecular weight cutoff of 3000, which excluded the bacteria from the internal volume but permitted the diffusion of the soluble form of the compound.

When enough sorbent was present to lower the concentration of PNP in solution to below 10 ng/ml, no mineralization of the compound by *Pseudomonas* sp. K was detected. Microbial populations in sediment which had not previously been exposed to PNP were also not able to mineralize the compound when concentrations in solution were decreased to below 10 ng/ml by sorption, but mineralization did occur when the bacteria had previously been exposed to 80 ng/ml of the compound in solution.

The results of the study show that sorption decreased the rate of biodegradation of organic compounds, that the rate of degradation is related to the initial concentration of the compound in solution and the rate of its desorption, and that sorption can reduce the concentration of the compound in solution to a value so low that degradation does not occur.

Our further investigations were designed to inquire into the mechanism of utilization of sorbed organic molecules. For this purpose, biphenyl sorbed to polyacrylic beads was the test system. A microbial consortium mineralized biphenyl sorbed to polyacrylic beads faster than the slow rate at which much of the compound was desorbed. Pure cultures of bacteria isolated from the consortium mineralized biphenyl in solution but not the sorbed compound. However, combinations of two strains did degrade the molecule. The consortium did not reduce the surface tension in media containing sorbed biphenyl or biphenyl in solution, and addition of synthetic and microbially produced surfactants to pure cultures did not result in utilization of sorbed biphenyl by cultures of isolates able to use the soluble molecule. Cells from the consortium that were attached to continuously washed beads degraded the substrate. It therefore appears that

bacteria may act on sorbed compounds without the necessity of an initial desorption and that the mechanism may involve cells attached to the particles rather than by the excretion of a surfactant.

Hydrophobic pollutants in soils, subsoils, and aquifers are sorbed to the solid phases of these environments. A number of means have been proposed for remediating such contaminated sites, but these procedures are generally expensive and sometimes result in the transfer of the toxicants to the atmosphere or to an absorbent that must itself then be treated or disposed of. Bioremediation is an attractive alternative to such technologies because it may be less expensive and results in the destruction of the polluting compounds. However, many sorbed hydrocarbons are resistant to biodegradation or are only metabolized slowly, as we report above.

In our previous studies, we showed that the nonionic alcohol ethoxylate surfactants Alfonic 810-60 and Novel II 1412-56 at 10  $\mu\text{g/g}$  of soil markedly increased the extent of biodegradation of phenanthrene in both a mineral and an organic soil; the stimulation was greater in the organic soil. Biphenyl mineralization in the mineral soil was not affected by either surfactant, but biodegradation in the organic soil was enhanced by Alfonic 810-60 at 100  $\mu\text{g/g}$ . In those early studies demonstrating enhancement of biodegradation by low concentrations of surfactants, we applied surfactants directly to the environmental sample containing the hydrocarbon. However, the site of contamination is typically below the surface and thus somewhat distant from the soil surface or injection wells bored into the surface materials, and the beneficial effect might not occur because the surfactant is either sorbed or degraded at locations in the overlying or adjacent soil. Hence, a study was conducted to determine whether Novel II 1412-56 added to the surface of Lima silt loam would enhance the biodegradation of phenanthrene and biphenyl present within the soil. Water containing the surfactant at concentrations of 10 and 100  $\mu\text{g/ml}$  was pumped through the soil. At 10  $\mu\text{g/ml}$ , Novel II 1412-56 markedly enhanced the rate and extent of phenanthrene mineralization and the extent but not the initial rate of biphenyl mineralization. The stimulation was less if the water added to the soil surface contained 100  $\mu\text{g}$  surfactant/ml. Addition of the surfactant at the two concentrations did not result in leaching of either phenanthrene or biphenyl, but products of the degradation were found in the soil leachate with or without the surfactant. These findings suggest that surfactants at low concentrations may be useful for in-situ bioremediation of sites contaminated with hydrophobic pollutants without causing movement of the parent compounds to groundwaters.

Sorption of organic chemicals to soil and sediments often entails an initially rapid and reversible process followed by a period of slow sorption occurring over weeks, months, or perhaps years, and the slow sorption leads to a chemical fraction that then resists desorption. The desorption-resistant fraction is often persistent in

natural environments. Polychlorinated biphenyls, pesticides, and halogenated aliphatic hydrocarbons have been found to exist in soils and sediments partially in a strongly sorbed, resistant form, and the size of this desorption-resistant fraction may increase dramatically with time as the chemical remains in the soil or sediment. The processes by which organic compounds become increasingly desorption-resistant in soils and sediments, sometimes termed chemical aging, are poorly understood. The importance of aging to the environmental fate of organic compounds is largely unexplored. As a result, a study was initiated to determine the effect of aging time in soils upon the biodegradability and extractability of organic compounds. Phenanthrene and PNP were chosen as test molecules because upon initial addition to soil they are readily biodegradable but differ markedly in hydrophobicity. The two compounds were aged in sterilized loam and muck, and bacteria able to degrade the compounds were then added to the soils. Increasingly smaller amounts of phenanthrene in the muck and PNP in both soils were mineralized with increasing duration of aging. Aging also increased the resistance of phenanthrene to biodegradation in nutrient-amended aquifer sand. The rate of mineralization of the two compounds in both soils declined with increasing periods of aging. The amount of phenanthrene and PNP added to sterile soils that was recovered by butanol extraction declined with duration of aging, but subsequent Soxhlet extraction recovered phenanthrene from the loam but not the muck. The extents of mineralization of phenanthrene previously incubated for up to 27 days with soluble or insoluble organic matter from the muck were similar. Less aged than freshly added phenanthrene was biodegraded if aggregates in the muck were sonically disrupted. The data show that compounds added to soil become increasingly more resistant with time to biodegradation and extraction.

Solvents and spilled or leaking fuels are often found in the form of nonaqueous phases in polluted environments. These nonaqueous-phase liquids (NAPLs) in soils or aquifers frequently are long-term sources of toxic contaminants in ground water. The transport and dissolution of NAPLs and their constituent solutes in the aqueous phase have been predicted or determined empirically, but data are scarce on biodegradation necessary to complement predictions of the fate of organic chemicals dissolved in solvents. Furthermore, because NAPLs concentrate hydrophobic chemicals in small areas, localized nutrient depletion may occur. The suppression of biodegradation of constituents of NAPLs would be accentuated if the NAPL were easily degraded because nutrients would be rapidly depleted. Indeed, the convective transport of  $O_2$  and other nutrients has been suggested to limit the bioremediation of NAPLs in aquifers. Because of the importance of NAPLs in determining the fate of organic compounds in polluted environments, we have investigated the role of NAPLs in affecting biodegradation of hydrophobic compounds and the mechanisms by which microorganisms acquire substrates that are present in NAPLs.

NAPLs may affect the rate of biodegradation by altering the availability of hydrophobic compounds to microorganisms. An *Arthrobacter* strain mineralized naphthalene and *n*-hexadecane dissolved in 2,2,4,4,6,8,8-heptamethylnonane. The extent of mineralization increased with greater volumes of solvent. Measurements under aseptic conditions of the partitioning of naphthalene into the aqueous phase from the solid phase or from heptamethylnonane showed that the rates were rapid and did not limit mineralization. The rate of mineralization of hexadecane was rapid, although partitioning of the compound into aqueous solution was not detected. The *Arthrobacter* sp. grown in media with or without heptamethylnonane did not excrete products that increased the aqueous solubility of naphthalene and hexadecane. Measurements of the number of cells in the aqueous phase showed that the *Arthrobacter* sp. attached to the heptamethylnonane-water interface, but attachment was evident even without a substrate in the heptamethylnonane. Tests with small inocula of the *Arthrobacter* sp. demonstrated that at least a portion of naphthalene or hexadecane dissolved in heptamethylnonane was degraded by cells attached to the solvent-water interface. The cells did not adhere in the presence of 0.1% Triton X-100. The surfactant prevented mineralization of the hexadecane initially dissolved in heptamethylnonane, but it increased the rate and extent of mineralization of naphthalene initially dissolved in heptamethylnonane. The data show that organic solvents into which hydrophobic compounds partition affect the biodegradation of those compounds and that attachment of microorganisms to the organic solvent-water interface may be important in the transformation.

A study was then conducted to determine the mineralization in soil and subsoil of hydrophobic organic compounds present in several NAPLs. When present in soil in some NAPLs, di(2-ethylhexyl) phthalate (DEHP) was not appreciably mineralized, phenanthrene was slowly transformed after an acclimation phase, and hexadecane and naphthalene were biodegraded rapidly. The extent of suppression of biodegradation of test compounds varied with different solvents as NAPLs. The rate of mineralization in subsoil of phenanthrene dissolved in some NAPLs was very low, but additions of N and P enhanced the degradation. The addition of N and P to soil did not increase the mineralization of DEHP dissolved in NAPLs. The existence of a NAPL in polluted environments may thus markedly affect the susceptibility of organic compounds to biodegradation.

In an extension of the tests with environmental samples, biodegradation of phenanthrene, biphenyl, or di(2-ethylhexyl) phthalate initially present in a variety of NAPLs was found to be slow in samples of soil and aquifer solids. The NAPLs were hexadecane, dibutyl phthalate, 2,2,4,4,6,8,8-heptamethylnonane, cyclohexane, commercial oils, crude oil, creosote, and kerosene. Slurrying the soil or aquifer solids markedly enhanced the rate and extent of mineralization of the test compounds initially in many of the NAPLs. Both the low rate and the extent of mineralization of the three compounds initially in dibutyl phthalate in soil slurries and of di(2-ethylhexyl) phthalate in heptamethylnonane present in slurries of

aquifer solids were increased by inoculation of acclimated microbial cultures. Increasing the NAPL volume decreased phenanthrene biodegradation in soil, but the effect of larger NAPL volume could be alleviated by slurring and inoculation. The rate or extent of mineralization in aquifer slurries of di (2-ethylhexyl) phthalate initially in some NAPLs was increased by addition of N and P, and inoculation further enhanced the degradation.

We report above that the addition of surfactants to environmental samples contaminated with hydrophobic compounds is a possible means of increasing the bioavailability of these compounds and to facilitate their biodegradation. Thus, the addition of low concentrations of nonionic surfactants was observed to stimulate the biodegradation of phenanthrene and biphenyl in soil. It is possible, however, that a hydrophobic compound trapped within surfactant micelles would not be available for microbial uptake and that bacteria use only the compound in the water phase. Therefore, a study was conducted to investigate the possible adverse effects of surfactant addition on the biodegradation of two hydrophobic compounds, biphenyl and phenanthrene.

Several of the surfactants tested were toxic to the test bacteria and prevented biodegradation of biphenyl and phenanthrene at concentrations below the critical micelle concentration (CMC). The rate of biodegradation was reduced at a surfactant concentration above but not below the CMC only when the test bacterium grew on biphenyl in the presence of Triton X-100. This decrease was correlated with the CMC and was more pronounced at a biphenyl concentration of  $0.2 \mu\text{g/ml}$  than  $2.0 \mu\text{g/ml}$ . Such a correlation was not observed when bacteria grew on  $1.0 \mu\text{g}$  glutamate per ml, even though the rate of glutamate degradation was reduced by Triton X-100. Analysis of the data with a mathematical model suggested that the experimental observations were probably not the consequence of the unavailability to bacteria of biphenyl within Triton X-100 micelles.

Information on the kinetics of biodegradation is important to predict the changes in concentration of chemicals with time as well as to understand how microorganisms transform organic compounds that are sorbed. Kinetic data allow the prediction of whether pollutants will be present at hazardous levels in the future or by the time they are transported to areas containing populations of sensitive species. Although attention has been given recently to the kinetics of biodegradation of sorbed chemicals, the topic remains relatively unexplored. Because clays are major sorbents in soils and sediments, a study was designed to investigate the kinetics of biodegradation of a compound sorbed by montmorillonite.

The first model we developed requires inputs in the form of parameters provided by separate measurements of the adsorption isotherm under aseptic conditions and the rate of biodegradation in the absence of sorbent. The only parameter that must be provided by the data to be simulated is the fraction of

carbon from the test compound that is incorporated into microbial cells or retained by the sorbent. The model was tested with benzylamine as test compound, a bacterial isolate able to mineralize the chemical, and montmorillonite as sorbent. Benzylamine mineralization by high cell densities of the bacterium was slower in the presence of montmorillonite than in the absence of the clay. Experimental curves for bacterial mineralization of benzylamine in well-dispersed suspensions of montmorillonite were reproduced by the model with standard deviations of usually less than 4%.

To quantitatively describe biodegradation under conditions of substrate limitation, a process-based deterministic model called the diffusion-sorption-biodegradation (DSB) model also was developed to describe the biodegradation of an organic chemical in the presence of spherical aggregates. Diffusion is described by Fick's second law, sorption by a linear isotherm, and biodegradation by one of various rate equations including first-order kinetics. Sensitivity analyses were performed to determine the dependence of a chemical's half-life on soil physical and chemical properties. Half-lives in the presence of aggregates were substantially greater than equivalent half-lives in the absence of aggregates when the sorption partition coefficient was  $\geq 3.2 \text{ dm}^3\text{kg}^{-1}$  and the aggregate radius was  $\geq 0.32 \text{ cm}$ . The two-compartment model provided a better fit than did a simple first-order model to simulations of the biodegradation of a sorbing chemical in the presence of large (0.25-cm radii) aggregates, whereas there was no difference between the two models for aggregates of much smaller radii. When simple first-order kinetics was assumed without accounting for diffusion and sorption, long-term extrapolation of chemical persistence in the presence of aggregates overestimated the degradation of a chemical with a sorption partition coefficient of  $10 \text{ dm}^3\text{kg}^{-1}$ . Considering that many organic pollutants sorb strongly and numerous soils have some degree of aggregate structure and organic matter, taking into account the physical and chemical processes affecting a chemical's concentration may greatly improve kinetics models of biodegradation in soil.

To test whether the kinetics of biodegradation in the presence of aggregates could be described by explicitly accounting for chemical diffusion, studies were conducted in well-defined experimental systems. The kinetics of biodegradation of low concentrations of  $^{14}\text{C}$ -labeled phenol and glutamate by *Pseudomonas* sp. strain K in buffer containing spherical aggregates of kaolinite that exclude bacteria were significantly different from the kinetics measured in the absence of aggregates. Both the biodegradation rate and the percentage of the initial compound degraded were lower in the presence of aggregates than in their absence. Using measurements of biodegradation, diffusion rates, and physical properties of the experimental system as input parameters, the DSB model simulated the biodegradation of phenol and glutamate originating inside aggregates. The model also simulated the initial period of biodegradation of glutamate in an experimental system containing gel-exclusion chromatography beads. Clay aggregates reduced

the concentration of available PNP sufficiently to lower the apparent rate constant for its biodegradation.

The rate of biodegradation of organic compounds in soil is often faster following the second than the first addition of the chemical. Several hypotheses have been proposed to explain this phenomenon of enhanced degradation; growth of the population, induction of enzymes, and selection of new metabolic capabilities produced by genetic change. Because of the large number of variables potentially involved in enhanced biodegradation, it is difficult to determine the contribution of a change in an individual factor, such as an increase in the growth rate, to the enhancement. However, kinetic models permit a quantitative determination of the degree of dependence of the rate of biodegradation on each of the parameters controlling the rate; thus, models may provide new insight into the phenomenon of enhanced degradation.

For our study of the kinetics of enhanced biodegradation, mineralization of 0.01, 0.1, 5.0, and 50 mg of carbonyl-<sup>14</sup>C-labeled 2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate (carbofuran) per kilogram of soil was measured in soil that had not been exposed to the pesticide and in soil that had been previously treated with the same concentrations of carbofuran. The stimulation in mineralization rate as a result of previous treatment of the soil with carbofuran was not the result of a substantial increase in the size of the microbial population able to use the compound, as indicated by most probable number counts. Of the Monod (single-substrate) and dual-substrate models of biodegradation kinetics, model I of the dual-substrate models provided the best fit to all curves of mineralization of carbonyl-labeled carbofuran. The fact that model I fit the data supports the hypothesis that the microorganisms mineralizing the carbonyl-labeled molecule do not grow at the expense of the methylcarbamate moiety.

## PUBLICATIONS

Kinetics of Bacterial Degradation of Benzylamine in a Montmorillonite Suspension, by Michael E. Miller and Martin Alexander. *Environ. Sci. Technol.* 25:240-245 (1991).

Kinetic Analysis of Enhanced Biodegradation of Carbofuran, by Kate M. Scow, Rebecca R. Merica and Martin Alexander. *J. Agric. Food Chem.* 38:908-912 (1990).

Inoculum Size as a Factor Limiting Success of Inoculation for Biodegradation, by Mohamed A. Ramadan, Ossama M. El-Tayeb and Martin Alexander. *Appl. Environ. Microbiol.* 56:1392-1396 (1990).



- Factors Affecting the Microbial Degradation of Phenanthrene in Soil, by V. B. Manilal and Martin Alexander. *Appl. Microbiol. Biotechnol.* 35:401-405 (1991).
- Biodegradation of Sorbed Chemicals in Sediments and Model Systems, by Rochelle Araujo. Ph.D. Thesis. Cornell Univ., Ithaca, N.Y. (1990).
- Effect of Diffusion on the Kinetics of Biodegradation: Experimental Results with Synthetic Aggregates, by Kate M. Scow and Martin Alexander. *Soil Sci. Soc. Am. J.* 56:128-134 (1992).
- Biodegradation by an *Arthrobacter* Species of Hydrocarbons Partitioned into an Organic Solvent, by Rebecca A. Efroymsen and Martin Alexander. *Appl. Environ. Microbiol.* 57:1441-1447 (1991).
- Influence of Calcium, Iron, and pH on Phosphate Availability for Microbial Mineralization of Organic Chemicals, by B. K. Robertson and Martin Alexander. *Appl. Environ. Microbiol.* 58:38-41 (1992).
- Effect of a Non-Ionic Surfactant Added to the Soil Surface on the Biodegradation of Aromatic Hydrocarbons Within the Soil, by Boris N. Aronstein and Martin Alexander. *Appl. Microbiol. Biotechnol.* 39:386-390 (1993).
- Effect of Diffusion and Sorption on the Kinetics of Biodegradation: Theoretical Considerations, by Kate M. Scow and John Hutson. *Soil Sci. Soc. Am. J.* 56:119-127 (1992).
- Selecting Inocula for the Biodegradation of Organic Compounds at Low Concentrations, by Miriam A. Pahm and Martin Alexander. *Microb. Ecol.* 25:275-286 (1993).
- Bacterial Transport and Degradation in a Loamy Soil and Sand, by Medha Devare. M.S. Thesis, Cornell University, Ithaca, N.Y. (1994).
- Bacterial Transport and Phenanthrene Biodegradation in Soil and Aquifer Sand, by Medha Devare and Martin Alexander. *Soil Sci. Soc. Am. J.*, in press (1995).
- The Effects of Nutrient Limitation and Predation on the Biodegradation of Substrates in Mixtures, by Wendy S. Steffensen. M.S. Thesis, Cornell University, Ithaca, N.Y. (1994).
- Enhanced Mineralization of Organic Compounds in Nonaqueous-Phase Liquids, by Michael P. Labare and Martin Alexander. *Environ. Toxicol. Chem.* 14:257-265 (1995).

Biodegradation in Soil of Hydrophobic Pollutants in Nonaqueous-Phase Liquids (NAPLs), by Rebecca A. Efroymson and Martin Alexander. Environ. Toxicol. Chem. 13:405-411 (1994).

Biodegradation of Hydrophobic Compounds in the Presence of Surfactants, by Francois Roch and Martin Alexander. Environ. Toxicol. Chem., in press (1995).

Effect of Aging of Chemicals in Soil on Their Biodegradability and Extractability, by Paul B. Hatzinger and Martin Alexander. Environ. Sci. Technol., 29:537-545 (1995).

Role of Competition for Inorganic Nutrients in the Biodegradation of Mixtures of Substrates, by Wendy S. Steffensen and Martin Alexander. Submitted for publication (1995).

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#### **INVENTIONS**

None